Selbyana 16(2): 147–149

THE IMPORTANCE OF TISSUE CULTURE TECHNIQUE FOR CONSERVATION OF ENDANGERED BRAZILIAN BROMELIADS FROM ATLANTIC RAIN FOREST CANOPY

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ABSTRACT. Seeds were used for establishment of a sterile culture of *Vriesea fosteriana* and *Vriesea hieroglyphica,* two endangered ornamental Brazilian bromeliads. Large number of plantlets could be obtained from embryos of *Vriesea hieroglyphica* when cultivated in Knudson basal medium. In contrast, for *Vriesea fosteriana* the addition of hormones was required to produce protuberances from which buds were formed. In this process, new leafy shoots can be formed continuously, providing explants to continue the multiplication. Entire leaves removed from aseptically-grown seedlings of Vriesea fosteriana were also used as a source of explants. After twenty days of culture, protuberances were formed at the basal part of them and shoot-buds appeared after one month of culture. For rooting, two different auxins were necessary depending on the species. The rooted shoots were planted in pots and grown in the greenhouse. All plants survived and looked phenotypically normal. This methodology provided a large number of plants, preserving the genetic diversity of the species. This feature is essential for any replacement program that makes use of micropropagated plants.

INTRODUCTION

The original extent of the Atlantic rain forest is unclear but it is estimated it covered more than 350,000 square kilometers. Nowadays there is not a homogenous forest, and the remaining fragments do not exceed five percent of the original area. In the State of São Paulo, the main part of this forest has been burned to make way for large-scale plantations, such as coffee, and other crops like cotton and sugar cane. In the States of Minas Gerais, Rio de Janeiro and Espírito Santo much of their forest was lost in the production of charcoal to supply energy for the metalurgical industry. Furthermore, the most populated Brazilian cities are located on the Atlantic coastal region making it still more difficult for the preservation of the remnants (Por 1992).

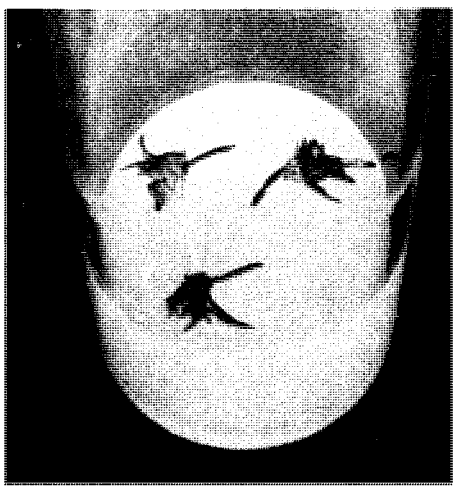
The canopy of the Atlantic rain forest is in general 20–30 m high. The epiphytic flora is very rich in species of bromeliads, orchids, ferns and other plants. At present some bromeliads are considered to be endangered. This is the case of *Vriesea fosteriana* L.B. Smith and *Vriesea hieroglyphica* (Carrière) Morren (Leme & Marigo 1993). Both species, found in the canopy of the Southeastern Brazilian Atlantic rain forest, present highly decorative leaves. That is the reason they are intensively collected throughout the year for the illegal trade. Like other *Vriesea* species, they develop very slowly from seeds and produce few offshoots after the flowering period (Mekers 1977). The natural methods of propagation are unable to produce the large numbers of plants, over a short term, needed for commercial purposes.

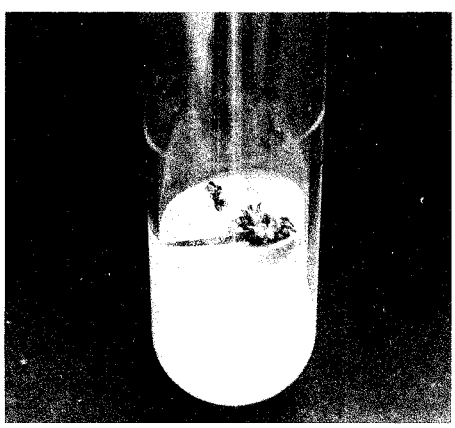
In this sense, plant propagation by tissue culture has many advantages. Generally, much higher multiplication rates can be obtained by *in vitro* methods than by conventional propagation. For most ornamental plants the starting material is a specific plant with a particular phenotype (Debergh & Zimmerman 1991). However, if the aim is genetic conservation, the initiation of the *in vitro* culture should be done through seeds collected from as wide a range of genotypes as possible. The aseptic seedlings obtained through *in vitro* seed germination can give rise directly to new plants or they can be used as an explant source for leaves. This strategy is very suitable for endangered species since it is difficult to obtain a large number of mother-plants to start a shoot-tip culture. This method also avoids the difficulties of disinfection of shoot-tips and axillary buds excised from wild bromeliads (Zimmer & Pieper 1976). Another advantage derived from the use of this method, is closely related to the possibility of obtaining the desirable genetic diversity (Roger 1984), since cultures can be easily and rapidly established using plants germinated from the aseptic seed sowing.

MATERIAL AND METHODS

Seeds of *Vriesea fosteriana* L.B. Smith were collected from plants in the Atlantic rain forest of Espírito Santo State and the seeds of *Vriesea hieroglyphica* (Carrière) Morren were harvested from plants in the "Serra do Mar", São Paulo State.

The seeds were disinfected by washing with





70% ethanol for two min followed by a 50% chlorine bleach for 40 min. After that, the seeds were washed up to five times with sterile distilled water and then transferred to the culture medium.

The basal nutrient medium used for *in vitro* seed germination and seedling culture was the Knudson medium (1946) in gelled or liquid state. In the experiments on seedling multiplication and leaf explant regeneration, the Knudson medium was supplemented with cytokinin (BA-benzylaminopurine) and/or auxin (NAA-naphthaleneacetic acid) at various concentrations (Mercier & Kerbauy 1992, 1994).

For root inducement, isolated shoots of approximately 2 cm in height were transferred to a gelled nutrient medium containing only NAA (0.2 mgl^{-1}), in the case of *V. fosteriana* or IBA (indole butyric acid) at a concentration of 5.0 mgl^{-1} for *V. hieroglyphica* species.

Results and Discussion

Seed germination and early development

The germination of the seeds took place up to two weeks after sowing. The seedlings showed a better growth rate on gelled Knudson medium than in liquid one. For *V.* hieroglyphica, it must be pointed out that gelled Knudson medium without any growth regulators stimulated the embryos to give rise to 3–7 plantlets instead of only one seedling (FIGURE 1), as would be expected in the normal process of germination (Mercier & Kerbauy 1994). Probably, this result represents a case of polyembryony induced by the culture medium.

Multiple shoot regeneration from seedlings

For *V. fosteriana* the transference of the seedlings from a gelled Knudson basal medium to the same medium supplemented with various concentrations of auxin and cytokinin gave rise to protuberances (FIGURE 2) from which buds

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FIGURE 1. Clusters of plantlets from *in vitro* seed germination of *Vriesea hieroglyphica*, after 3 months of culture on gelled Knudson medium without any hormones.

FIGURE 2. Clusters of protuberances from in vitro seed germination of *Vriesea fosteriana*, after 2 months of culture on gelled Knudson medium added with 0.5 mgl^{-1} NAA and 2.0 mgl^{-1} BA.

FIGURE 3. A shoot-bud cluster formed at the basal part of the leaf explant of *Vriesea fosteriana*, after 2 months of culture on gelled Knudson medium supplemented with 0.5 mgl^{-1} NAA and 2.0 mgl^{-1} BA.

were formed after three months of culture. The combination of 0.5 mgl^{-1} NAA and 2.0 mgl^{-1} BA induced the best results both in terms of percentage of regenerated buds (100%), and number of buds per explant (22.5 ± 2.0). Subsequent transfer to the same medium in the liquid state doubled the number of buds (Mercier & Kerbauy 1992). In this process, new leafy shoots can be formed continuously, providing explants to continue the multiplication. Despite the conspicuous results on adventitious bud regeneration of *V. fosteriana,* no effect of the aforementioned medium was observed on lateral bud development. This suggests that apical dominance seems to be involved.

In the case of *V. hieroglyphica,* it was shown that seedlings cultivated in the Knudson basal medium with added 2.0 mgl^{-1} BA alone or BA (2.0 mgl^{-1}) in combination with 0.5 mgl^{-1} NAA produced the best results of axillary bud development. The multiplication rate was seven shoots per seedling after six months of culture (Mercier & Kerbauy 1994).

Multiple shoot regeneration from leaf explants

Young leaves with a minimum length of 2 cm were detached from aseptically grown seedlings of *V. fosteriana* and re-cultured on a gelled Knudson medium added with NAA and BA. The optimum combination for bud regeneration was the same as described for the seedling explants. After 20 d of culture, protuberances were formed at the basal end of the leaves and shoot-buds appeared after one month of culture (FIGURE 3). The best rate was 15.2 ± 2.4 buds per leaf with a frequency of 50%. This system shows a high proliferative rate, making it possible to obtain a large number of *V. fosteriana* shoots every year (Mercier & Kerbauy 1992).

Rooting and planting out

NAA at concentration of 2.0 mgl^{-1} was used to promote root development of *V. fosteriana* species. After two months of culture, every shoot formed 3–4 roots. For *V. hieroglyphica* 5.0 mgl^{-1} of IBA was required to induce root formation and after four months, 50% of the shoots present 1–2 roots.

The plantlets of both species were transferred to pots containing a mixture of peat and vermiculite (1:1) and were grown in a greenhouse under natural day conditions. The rate of survival was 100% after a year.

Genetic stability

The adventitious bud regeneration in *V. fosteriana* produced 100% phenotypically normal plants after four years of culture in a greenhouse. The genetic uniformity of the shoots adventitiously regenerated depends on both the nature of the explant and the type and level of hormones used. The least differentiated organ tissue used with a hormonal balance adjusted to avoid callus formation much reduces production of mutated plantlets (Hussey 1984). Polyploidy, that occurs naturally during the differentiation process with a frequency of 90%, is the most common source of genetic aberration which might arise from the use of older tissues (D'Amato 1978).

For *V. hieroglyphica,* the axillary bud system also produced 100% true-to-type plants. Organized meristems have been shown to be the most genetically stable parts of the plant and a multiplication process based on axillary bud development is the method least likely to incur mutation (Hussey 1984).

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